

# Intravenous infusion of iron and tetrahydrofolate does not influence intrauterine uteroferrin and secreted folate-binding protein content in swine<sup>1</sup>

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**ABSTRACT:** The effect of exogenous iron and folate on reproductive performance in swine is equivocal. However, the effect of exogenous iron and folate on secretion of their respective uterine transport proteins has never been reported. Twenty gilts were infused (n = 5 per treatment) with either 1) saline, 2)  $\alpha$ -tocopherol, 3)  $\alpha$ -tocopherol plus iron citrate, or 4)  $\alpha$ -tocopherol plus tetrahydrofolate on d 11 to 14 of pregnancy. Intravenous infusion of iron citrate and tetrahydrofolate increased ( $P < 0.05$ ) plasma iron and folate, respectively,

for 6 to 8 h after treatment. Treatments had no effect on uterine content of uteroferrin or secreted folate-binding protein in uterine flushings obtained on d 15 of pregnancy. These data suggest that uterine secretion of uteroferrin and secreted folate-binding protein are not influenced by plasma levels of iron and folate, respectively, and may provide an explanation for the equivocal effect of iron and folate treatment on reproductive performance in gilts.

Key Words: Folic Acid, Pigs, Pregnancy, Uterus

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## Introduction

Recent experiments indicate that intrauterine crowding has a negative impact on erythropoiesis (see Vallet, 2000 for review). This decrease in fetal erythropoiesis could reduce fetal health and survivability. Iron and folate are both required for efficient erythropoiesis; thus, increased iron and folate delivery to the swine conceptus could improve erythropoiesis and increase litter size.

Neonatal pigs are iron-deficient (Ullrey et al., 1960), and many studies have been performed in which pregnant gilts or sows have been treated with exogenous iron to alleviate this problem and improve reproductive performance (Spruill et al., 1971; O'Connor et al., 1989; Guise and Penny, 1990). No improvement in litter size and equivocal effects on neonatal iron supply were obtained. These results are not surprising given the homeostatic mechanisms that regulate plasma iron (Hallberg, 1981; Huebers and Finch, 1987; Theil, 1987). Iron reaching the fetus is limited by gut uptake

(Hallberg, 1981), metabolism by the liver and other tissues (Huebers and Finch, 1987; Theil, 1987), and secretion rate of uteroferrin by the uterus (Roberts et al., 1986; Vallet et al., 1996, 1998b).

Effects of exogenous folate on litter size of swine have also been examined. Some studies reported increased litter size (Matte et al., 1984; Lindemann and Kornegay, 1989; Thaler et al., 1989), whereas others indicated no effect (Tremblay et al., 1989; Harper et al., 1994). As with iron, gut absorption, liver and other tissue metabolism (Steinberg, 1984; Shane and Stokstad, 1985), and uterine transport via a newly discovered folate-binding protein secreted by the uterus (Vallet et al., 1998a; 1999a, b) may limit availability of folate to the conceptus.

The objectives of the following experiment were to determine the effect of exogenous iron and folate treatment on uteroferrin and secreted folate-binding protein in the intrauterine environment during early pregnancy.

## Materials and Methods

Twenty white crossbred (1/4 Chester White, 1/4 Large White, 1/4 Yorkshire, 1/4 Landrace) gilts were observed daily for estrous behaviour and mated at 24-h intervals when detected in estrus. To bypass the influence of the gut and the liver on plasma iron and folate concentrations, the iron and folate were administered as iron citrate and tetrahydrofolate forms that are directly usable by the body tissues. Tetrahydrofo-

<sup>1</sup>Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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late was used instead of folic acid because it is the dominant form of folate circulating in plasma of swine (Natsuhori et al., 1991). Gilts were assigned to the following treatments ( $n = 5$  per group): 1) 20 mL of saline per day; 2) 1 mg  $\alpha$ -tocopherol in 20 mL saline; 3) 100 mg Fe Citrate plus 1 mg  $\alpha$ -tocopherol in 20 mL saline, and 4) 500  $\mu$ g tetrahydrofolate plus 1 mg  $\alpha$ -tocopherol in 20 mL saline. The dosages of iron and tetrahydrofolate were calculated to raise plasma concentrations two- to threefold, based on the concentrations normally found in plasma. Because free iron is a strong oxidant (Braugher et al., 1986), the  $\alpha$ -tocopherol was added to provide extra antioxidant activity to prevent detrimental oxidizing effects. Also, tetrahydrofolate is easily oxidized (Sasaki et al., 1996), and  $\alpha$ -tocopherol was added to prevent this spontaneous reaction. Jugular catheters were inserted into gilts on d 9 of pregnancy and treatments were given once daily (0800) from d 11 to 14. Briefly, gilts were anesthetized using sodium pentobarbital and anesthesia was maintained using halothane. A catheter guide was introduced through the skin and into the jugular vein and a plastic heparinized catheter was fed into the guide. The catheter guide was removed and the catheter was tunneled under the skin and exteriorized at the back of the gilt, where it was ligated in place. On d 14, blood samples were collected via the jugular catheter beginning just before infusion and at 2, 4, 6, 8, and 24 h after infusion. On d 15, catheters were removed and the gilts were slaughtered. The reproductive tracts were recovered and each uterine horn was flushed with 20 mL of 0.9% saline. Four to eight conceptuses per gilt were examined microscopically to determine the number of somites present. Uterine flushings were centrifuged and supernatants collected. Uterine flushings were measured for total protein, using the method of Lowry et al. (1951) with bovine serum albumin as the standard. Uteroferrin in the flushings was measured using an acid phosphatase assay as previously described (Vallet and Christenson, 1994). Retinol-binding protein and secreted folate-binding protein in the flushings were measured using previously validated specific radioimmunoassays (Vallet 1994; Vallet et al., 1999a). Plasma iron was measured using the method of Schade et al. (1954) and plasma folate was measured (Treatments 2 and 4) using a commercial radioligand assay (Quantaphase, BioRad Inc., Hercules, CA) validated in our lab for use in porcine plasma. Because of the expense of this assay, only samples from treatments 2 and 4 were measured. Retinol-binding protein and secreted folate-binding protein were each measured in a single assay; the intra-assay coefficients of variation were 16 and 6%, respectively. Inter- and intra-assay coefficients of variation for the plasma folate assays were 18 and 17%, respectively.

#### Statistical Analysis

Plasma concentrations of iron and folate were analyzed by analysis of variance using a model that in-

cluded effects of treatment, gilt within treatment, time after infusion, and the treatment  $\times$  time interaction. Individual treatment  $\times$  time interaction contrasts were used to more closely examine the effects of treatment. Protein, retinol-binding protein, acid phosphatase, and folate-binding protein concentrations in uterine flushings were multiplied by the recovery volume for each gilt to provide the total amount of each protein in the uterine flush. Average somite stage for each gilt was also calculated. These data were analyzed using analysis of variance and a model that included the effect of treatment. Treatment means were further analyzed using the orthogonal contrasts. For all traits except acid phosphatase, the contrasts used were 1) treatment 1 vs treatment 2 (effect of  $\alpha$ -tocopherol), 2) treatments 1 and 2 combined vs treatment 3 (effect of iron citrate treatment), and 3) treatments 1, 2, and 3 combined vs treatment 4 (effect of tetrahydrofolate treatment). For acid phosphatase, the contrasts used were 1) treatment 1 vs treatment 2, 2) treatments 1 and 2 combined vs treatment 4, and 3) treatments 1, 2, and 4 combined vs treatment 3. Total acid phosphatase, retinol-binding protein, and folate-binding protein were further analyzed using the above model along with total protein as a covariate.

#### Results

Plasma iron and folate least squares means are illustrated in Figure 1. Intravenous infusion of 100 mg Fe citrate increased ( $P < 0.05$ ) plasma iron concentrations for 6 h. Likewise, intravenous infusion of tetrahydrofolate increased ( $P < 0.05$ ) folate concentrations for 6 h. Thus, treatments were successful in raising the amount of iron and folate available to the tissues in the body.

Least squares means for total protein, total retinol-binding protein, total acid phosphatase, and total folate-binding protein in uterine flushings collected on d 15 of pregnancy along with the developmental stage of the conceptus (number of somites) are indicated in Table 1. The  $\alpha$ -tocopherol treatment increased ( $P < 0.05$ ) total protein and total retinol-binding protein but had no effect on total acid phosphatase or total folate-binding protein. Neither iron nor folate treatment had any effect on any of the proteins measured, whether or not total protein was used as a covariate. These results suggested that uterine uteroferrin and folate-binding protein secretion was unaffected by the availability of iron and folate, respectively. No effect of treatments on number of somites was observed.

#### Discussion

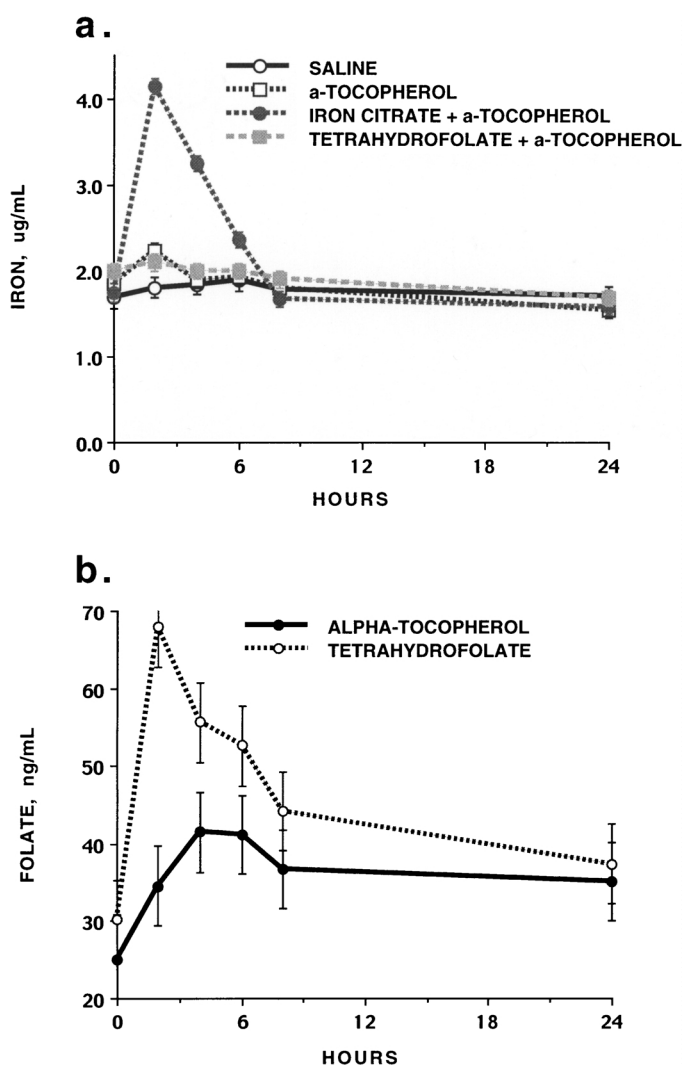
Results suggest that iron and folate treatment did not have an influence on secretion of uteroferrin or secreted folate-binding protein by the uterus during pregnancy. However, these data must be viewed with the following cautions. Given the sample sizes used,

and the variability in responses obtained, increases in folate-binding protein and uteroferrin of approximately 56 and 33% would have been necessary to reach statistical significance. Thus, smaller, undetected increases are still possible. Nevertheless, these results suggest that in normally fed gilts, the amount of uteroferrin and folate-binding protein in the intrauterine environment is controlled by factors other than availability of iron and folate, respectively. Thus, if one wishes to improve iron and folate delivery to the developing conceptus in swine, it may be more efficient to understand and then alter the mechanisms controlling production rate of these proteins.

These data suggest that increased delivery of exogenous iron and folate to the developing conceptus was inhibited by both systemic and uterine iron and folate

metabolism and provide an explanation for results indicating that exogenous iron and folate treatment have little effect on reproductive efficiency of swine. Infusion of iron citrate and tetrahydrofolate successfully raised plasma concentrations of iron and folate at 2 h after infusion, but homeostatic mechanisms returned the levels of both to normal concentrations by 8 h after infusion. The liver plays a role in regulating the concentrations of both iron and folate, and liver cells have receptors for both transferrin (Huebers and Finch, 1987) and folate (Van Hoozen et al., 1996). The return of plasma iron and folate concentrations to normal values after 8 h indicates the efficiency of this mechanism. These homeostatic mechanisms influence our ability to detect differences in secretion rates of uteroferrin and folate-binding protein and illustrate the inherent difficulty of improving nutrient delivery to the uterus.

Even though plasma levels of both iron and folate were temporarily elevated by the treatments, the uterine content of uteroferrin and folate-binding protein was unaffected. Thus, control of production of these proteins by the uterine endometrium may represent a further barrier to increased transport of iron and folate to the swine fetus. Previous results suggest that during early pregnancy, production rate of uteroferrin and folate-binding protein is controlled primarily by mechanisms associated with prolonged endometrial exposure to progesterone, with (uteroferrin, Vallet et al., 1998b) or without (folate binding protein, Vallet et al., 1998a, 1999a) modulation of the production rate by the conceptus. However, production of uteroferrin is not maximal at d 15 of pregnancy. It increases 20-fold from d 20 to d 40 (Vallet et al., 1996). There is also conceptus modulation of uteroferrin production during later pregnancy (Vallet et al., 1994; Vallet and Christenson, 1996). Thus, the timing of progesterone secretion by the corpus luteum and the conceptus both influence uterine protein production, but the details of the mechanisms that control rates of uteroferrin and folate-binding protein production are not understood well enough to enable increased production. Therefore, any exogenous iron or folate that escapes systemic mechanisms maintaining plasma concentrations of these two substances may still be blocked from reaching the fetus due to controls governing uterine uteroferrin and secreted folate-binding protein production. The most efficient way to increase iron and folate delivery to the developing swine conceptus may be to understand and manipulate the intrauterine mechanisms controlling uteroferrin and folate-binding protein production.



**Figure 1.** Plasma iron (a) and folate (b) least squares means  $\pm$  SEM for gilts treated with saline,  $\alpha$ -tocopherol,  $\alpha$ -tocopherol and iron citrate, and  $\alpha$ -tocopherol and tetrahydrofolate are illustrated. Iron citrate treatment raised plasma iron ( $P < 0.05$ ) until 8 h after injection. Tetrahydrofolate raised plasma folate concentrations ( $P < 0.05$ ) until 8 h after injection.

## Implications

Under the conditions used in this experiment, results suggest that intravenous administration of iron and folate does not increase uteroferrin or folate-binding protein concentrations in the uterus, despite tem-

**Table 1.** Least squares means  $\pm$  SEM for total protein, total retinol-binding protein, total acid phosphatase (uteroferin), total folate-binding protein, and somite stage, and total acid phosphatase; and total folate-binding protein after fitting total protein as a covariate in uterine flushings of d-15 pregnant gilts after treatment with saline,  $\alpha$ -tocopherol ( $\alpha$ -TOC), iron citrate (IC), and tetrahydrofolate (THF)

Item	Saline <sup>a</sup>	$\alpha$ -TOC	IC + $\alpha$ -TOC	THF + $\alpha$ -TOC
Total protein, mg <sup>b</sup>	185 $\pm$ 30	284 $\pm$ 27	253 $\pm$ 27	203 $\pm$ 27
Total retinol-binding protein, mg <sup>b</sup>	12.6 $\pm$ 1.8	17.8 $\pm$ 1.6	13.8 $\pm$ 1.6	15.1 $\pm$ 1.6
Total acid phosphatase, $\mu$ mol Pi/min	713 $\pm$ 83	929 $\pm$ 74	776 $\pm$ 74	710 $\pm$ 74
Total folate-binding protein, $\mu$ g	282 $\pm$ 54	255 $\pm$ 48	244 $\pm$ 48	363 $\pm$ 48
Number of somites	8.5 $\pm$ 2.4	9.6 $\pm$ 2.1	7.8 $\pm$ 2.1	4.2 $\pm$ 2.1
Adjusted for total protein				
Total acid phosphatase, $\mu$ mol Pi/min	709 $\pm$ 86	933 $\pm$ 77	777 $\pm$ 77	709 $\pm$ 77
Total folate-binding protein, $\mu$ g <sup>a</sup>	278 $\pm$ 56	259 $\pm$ 50	246 $\pm$ 50	360 $\pm$ 50

<sup>a</sup>Number of observations was 4 for saline-treated gilts and 5 for all other treatments.

<sup>b</sup>Mean for  $\alpha$ -TOC-treated gilts was greater than that for saline-treated gilts ( $P < 0.05$ ).

porarily increasing the amount of both substances in the blood. These results may provide reasons why iron and folate treatments do not result in an improvement in reproductive performance. Improvements in delivery of iron and folate to the developing swine conceptus may require methods that alter the secretion rate of uteroferin and folate-binding proteins. Development of methods to improve iron and folate transport to the conceptus may benefit overall development and fetal erythropoiesis and, therefore, may lead to increased litter size.

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